A mutation in the 3′-UTR of the HDAC6 gene abolishing the post-transcriptional regulation mediated by hsa-miR-433 is linked to a new form of dominant X-linked chondrodysplasia

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A family with dominant X-linked chondrodysplasia was previously described. The disease locus was ascribed to a 24 Mb interval in Xp11.3–q13.1. We have identified a variant (c.∗281A>T) in the 3′ untranslated region (UTR) of the HDAC6 gene that totally segregates with the disease. The variant is located in the seed sequence of hsa-miR-433. Our data showed that, in MG63 osteosarcoma cells, hsa-miR-433 (miR433) down-regulated both the expression of endogenous HDAC6 and that of an enhanced green fluorescent protein-reporter mRNA bearing the wild-type 3′-UTR of HDAC6. This effect was totally abrogated when the reporter mRNA bore the mutated HDAC6 3′-UTR. The HDAC6 protein was found to be over-expressed in thymus from an affected male fetus. Concomitantly, the level of total α-tubulin, a target of HDAC6, was found to be increased in the affected fetal thymus, whereas the level of acetylated α-tubulin was found to be profoundly decreased. Skin biopsies were obtained from a female patient who presented a striking body asymmetry with hypotrophy of the left limbs. The mutated HDAC6 allele was expressed in 31% of left arm-derived fibroblasts, whereas it was not expressed in the right arm. Overexpression of HDAC6 was observed in left arm-derived fibroblasts. Altogether these results strongly suggest that this HDAC6 3′-UTR variant suppressed hsa-miR-433-mediated post-transcriptional regulation causing the overexpression of HDAC6. This variant is likely to constitute the molecular cause of this new form of X-linked chondrodysplasia. This represents to our knowledge the first example of a skeletal disease caused by the loss of a miRNA-mediated post-transcriptional regulation on its target mRNA.

INTRODUCTION

About 400 genetic bone disorders have been described, 215 of which have been associated with one or more of 140 different genes (1). Diseases of the endochondral skeleton, called chondrodysplasias, are caused by defects in the differentiation and proliferation of chondrocytes or in the extra cellular matrix of the cartilage. The genes involved in chondrodysplasias code for extracellular matrix proteins, signalling factors, transcription factors or components of metabolic pathways. The signalling pathways involved in chondrodysplasias are the fibroblast growth factor receptors, the parathyroid

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hormone-related peptide, the Hedgehog pathway, the bone morphogenetic proteins and the Wnt pathways. Various metabolic anomalies, such as sulphation and sterol metabolism defects, also cause chondrodysplasias.

Chondrodysplasias constitute an extraordinarily complex ensemble of clinically heterogeneous diseases, the molecular bases of which are being increasingly understood with the discovery of the causative genes. It is not possible to provide here an extensive description of the various diseases and genes involved. This can best be found in the review by Superti-Furga and Unger (1), supported by the Online Mendelian Inheritance in Man catalogue.

We recently described (2) a new form of X-linked dominant chondrodysplasia (Fig. 1A). The phenotype in males is severe

Figure 1. Identification of a variant in the 3′-UTR of the HDAC6 gene in a family with X-linked dominant chondrodysplasia. (A) Pedigree of the family. (B) Electropherogram showing the presence of the HDAC6 3′-UTR A>T variant (arrow) in the heterozygous and hemizygous state in individuals II7 and III14, respectively. (C) Schematic representation of the sequence alignment of the hsa-miR-433 with nucleotides 4004–4027 of both the wild-type (WT) and variant HDAC6 mRNA. The seed region is shown in blue. The HDAC6 3′-UTR A>T mutation is shown in red.
and associates platyspondyly, rhizomelic shortening of the members, specific brachydactyly, hydrocephaly, facial dysmorphism and microphthalmia. The phenotype in heterozygous females is less severe and comprises short stature, body asymmetry and moderate mental retardation. This phenotype is distinct from that observed in other chondrodysplasias, namely achondrogenesis, thanatophoric dysplasia, atelosteogenesis and spondyloepimetaphyseal dysplasia. It is also different from that of CDPX1, CDPX2 and CHILD syndrome, the other X-linked types of chondrodysplasia. The presence of hydrocephaly in some male cases prompted us to test the L1CAM gene, but no mutation was found. A deletion of the SHOX gene was also excluded.

A genetic linkage analysis showed that the disease locus mapped within a 24 Mb interval spanning the pericentromeric region Xp11.3–q13.1 (Lod score = 3.30). As EBP (mutations of which cause CDPX2) lies in the interval of interest, a mutation scanning of this gene was performed, but did not display any anomaly. This correlates with biological data showing that 8-dehydrocholesterol and 8(9)-cholestanol levels were normal in the patients tested, whereas they are elevated in patients with CDPX2.

The form of chondrodysplasia observed in this family is therefore distinct from CDPX2 both at the clinical and biological levels and appears as a new disease entity.

We now set up to identifying the molecular basis of this disorder and identified as the probable cause a nucleotide substitution in the 3′ untranslated region (UTR) of the HDAC6 (Histone deacetylase 6) transcript. This mutation lies in the seed sequence of micro RNA-433 (hsa-miR-433). The functional analyses we have performed showed that the mutation totally abolishes the post-transcriptional regulation of HDAC6 expression by hsa-miR-433 (miR433) and results in the overexpression of the HDAC6 protein in tissues from affected patients.

RESULTS

Search for a gene dosage anomaly and sequencing analysis

In an attempt to narrow down the region in which the disease locus may reside, we have performed an array-CGH analysis with DNA from one female carrier (II7) (Fig. 1) and one male fetus (III14) using both 44K (II7) and 244K (II7 and III14) microarrays from Agilent Technologies. No dosage anomaly was identified, thus suggesting that no deletion or duplication of a chromosomal segment was involved in the pathogenesis.

A genetic linkage analysis performed using X-linked microsatellite polymorphic markers showed that the disease locus mapped within a 24 Mb interval framed by markers DXS8054 and DXS1275, thus spanning the pericentromeric region Xp11.3–q13.1 (Lod score = 3.30). This 24 Mb interval contains 80 genes, five of which stand out as possible candidates. BMP15 is a member of the bone morphogenetic proteins family. CHST7 is a carbohydrate sulphotransferase, a family of genes that comprises CHST3, mutations of which cause spondyloepiphyseal dysplasia (Omani type). EBP is mutated in X-linked recessive chondrodysplasia punctata type 2 (CDPX2). GSPT2 (G1 to S phase transition 2) encodes a protein involved in the control of the cell cycle that is expressed in brain and bones. TIMP1 (tissue inhibitor of metalloproteinase 1, also called collagenase inhibitor), is an inhibitor of metalloproteinase, some of which degrade collagen from the extracellular matrix, such as MMP2 that is mutated in multicentric osteolysis, nodulosis and arthropathy. EBP (involved in CDPX2) was already analysed previously and did not show any mutation. No point mutation was found by sequencing the exons of the four remaining genes in individuals I17 and III14. No gene dosage anomaly was identified by Quantitative Multiplex Fluorescent-PCR (3) in these genes either.

We then sequenced the exons of 31 additional genes, and identified a variant, c.281A>T, in the HDAC6 gene that encodes the Histone Deacetylase 6 protein (Fig. 1B).

Characterization of the HDAC6 3′-UTR variant

This variant lies in exon 29 of HDAC6, 281 bp after the TAA translation termination codon, i.e. in the 3′-UTR of the gene. The HDAC6 variant was found in all affected females (I1, I11, I12, II7, II11, II15 in the heterozygous state) and in males (II4, IV1) but not in the unaffected individuals (II3, II4, II5, II12, II13), thus displaying complete co-segregation with the disease in agreement with an X-linked dominant mode of inheritance. This variant is not described in the SNP databases and was not found in 100 control individuals tested (50 males and 50 females).

Considering the possibility that this 3′-UTR variant may have an effect on the regulation of HDAC6 expression, various in silico analyses were undertaken. Bioinformatics tools on http://www.targetscan.org/ (4–6) and http://microrna.sanger.ac.uk/ (7–10) showed that the c.281A>T variant resides inside a sequence matching the seed of mature hsa-miR-433 (miR433) (5′UAUAUAUG3′) (Fig. 1C). The predicted hsa-miR-433 site in HDAC6 3′-UTR is a 7mer-m8 type and contains the miRNA seed (miRNA nucleotides 2–7; 5′UCAUAGUG3′) augmented by a match to miRNA nucleotide 8 (5). One rule for miRNA-target matching is a perfect and contiguous match to miRNA nucleotides 2–7 representing the seed (5). This short sequence is necessary to the biological and functional property of the miRNAs and any mismatch in that sequence greatly affects gene expression regulation (11). Perfect match to the miRNA 3′ end, particularly between bases 13 and 16, is also important to stabilize the interaction between the miRNA and its target mRNA, which is the case here (Fig. 1C). However, the c.281A>T variant changes the predicted miRNA site from 5′AUCAUGGA3′ to 5′AUCUAGUGA3′ (Fig. 1C). Thus the variant introduces a mismatch at target position matching miRNA nucleotide 5 resulting in a site that does not perfectly match anymore the seed of hsa-miR-433. Therefore, this prompted us to determine whether HDAC6 mRNA could be a biological target of hsa-miR-433 in cells and to evaluate the consequence of this nucleotide substitution on HDAC6 expression.

Hsa-miR-433 controls HDAC6 expression in MG63 cells

To evaluate the functional property of hsa-miR-433 on HDAC6 expression, human osteoblast MG63 cells were
transfected with hsa-miR-433, anti-hsa-miR-433, both or none of them (mock transfected cells). Then HDAC6 expression was monitored by western blotting using total protein extracts. A significant decrease of the HDAC6 protein level was observed in cells transfected with hsa-miR-433 in comparison to the mock transfected cells (Fig. 2A and B, compare lanes 1 and 2) \( (P = 0.03; n = 3) \). The level of hsa-miR-433 expression in each transfection is shown in Figure 2C (means of two experiments). When cells were transfected with both hsa-miR-433 and anti-hsa-miR-433, the amount of HDAC6 was similar to that of mock transfected cells (Fig. 2A and B, compare lanes 2 and 4), demonstrating that the regulatory effect of hsa-miR-433 was neutralized by the corresponding anti-miR. However, HDAC6 expression in MG63 cells transfected with the anti-ha-miR-433 alone was not significantly different from that in mock transfected cells (Fig. 2A and B). This result was surprising since one might expect to see an increase of HDAC6 expression in this condition (neutralization of the endogenous hsa-miR-433). One possible explanation is that, when used alone, the anti-hsa-miR-433 was unable to enter into the cells by transfection or to recognize the endogenous hsa-miR-433. These results strongly suggested that hsa-miR-433 can specifically inhibit HDAC6 expression in MG63 cells and that HDAC6 mRNA might be a biological target of hsa-miR-433. We therefore managed to evaluate the regulation of the c."281A>T variant on this post-transcriptional regulation of HDAC6 by hsa-miR-433 in cellulo. We also observed that hsa-miR-433 was expressed in chondrocytes, a tissue that is relevant to the disease analysed here (data not shown).

**Hsa-miR-433 controls HDAC6 expression by targeting the 3’-UTR and destabilizing the mRNA, and the c."281A>T variant abrogates this regulation**

To evaluate the post-transcriptional regulatory effect of hsa-miR-433 on HDAC6 expression and the impact of the c."281A>T variant on this potential regulation, a method, named FunREG (for functional, integrated and quantitative method to measure post-transcriptional regulations) and developed by some of us, was used (12).

The wild-type HDAC6 3’-UTR or its mutated counterpart was inserted downstream of the enhanced green fluorescent protein (eGFP)-coding region into the pTRIP-eGFP Lentiviral vector. The corresponding plasmids were called pTRIPeGFP3’H- DAC6WT and pTRIPeGFP3’HDAC6Var, respectively (Fig. 3A). A control lentiviral vector (pTRIP-eGFP-GLO, Fig. 3A) containing the rabbit β-globin 3’-UTR (generally used as a control in post-transcriptional studies; 13) was used as reference for the basal eGFP-transgene expression in the target cells (12). The three vectors were used to produce infectious lentiviral particles containing the corresponding transgenes. Then human osteoblast MG63 and HeLa cells which, respectively, express and do not express hsa-miR-433 (Fig. 3B) were transduced by the lentiviral particles at a multiplicity of infection (moi) of 2. At this moi, 40–45% of the cells expressed eGFP and only one copy of transgene was integrated per cell genome (12). Seven days post-transduction, expression of eGFP protein (referred to as P) was measured in the different cell line populations by flow cytometry. The average number of lentiviral transgene copies per cell (or transgene copy number, TCN) was measured by quantitative (q) PCR in each condition in genomic DNA extracted from the transduced populations using the two copies of the albumin gene as a reference. Then the P/TCN ratio, which corresponds to the quantity of eGFP protein produced per transgene, was calculated for each transgene. When comparing eGFP expression of each transgene with that of the referent eGFP-GLO, a variation in P/TCN ratio is indicative of a post-transcription regulation (12).

In HeLa cells, no significant difference in P/TCN ratio was observed when comparing eGFP expression from eGFP-WT or eGFP-Var to that from eGFP-GLO (Fig. 3C, bar charts of eGFP-GLO transgene being equal to 1 and data not shown).

**Figure 2. Hsa-miR-433 controls HDAC 6 expression in MG63 cells.** (A) Western blot analyses using total protein extracts from MG63 cells were performed using antibodies raised against the HDAC6 and GAPDH (internal control) proteins. The proteins are as indicated on the right and their corresponding molecular weights in kDa are shown on the left. Transfection conditions (use of Interferin reagent, presence of hsa-miR-433, anti-hsa-miR-433) are as indicated by + or – symbols below the blots. One representative of three independent experiments. (B) Bar graphs showing the amounts of HDAC6 protein normalized to GAPDH in the indicated conditions. Asterisk indicates statistical significance \( (P = 0.02; n = 5) \). (C) The level of hsa-miR-433 expression is indicated for each condition, normalized to RNU48 (mean values from two experiments).
In other words, HDAC6 3'-UTR did not seem to bear any post-transcriptional mechanisms in these cells. On the other hand, eGFP expression was significantly higher in MG63 cells transduced with the construction harbouring the variant HDAC6 3'-UTR than in those transduced with the wild-type 3'-UTR (Fig. 3C, P = 0.02; n = 5). These data indicated that in MG63 cells, the single A>T variant affects the post-transcriptional regulation conveyed by the 3'-UTR of HDAC6. As hsa-miR-433 was found to be expressed in MG63 cells but not in HeLa cells, and as no difference in eGFP expression was reported in HeLa cells, we concluded that in accordance with the results shown in Figure 2, this post-transcriptional regulation in MG63 cells was likely to be due to the endogenous hsa-miR-433.

To prove that the difference in post-transcriptional regulation observed in MG63 cells expressing either the eGFP-WT or eGFP-Var was indeed due to hsa-miR-433, functional analyses using a synthetic miRNA and the FunREG method were undertaken. To increase the resolution of the measurements by flow cytometry, MG63 cells expressing each eGFP transgene were FACS-sorted in order to obtain a population with up to 90% eGFP positive cells (data not shown). Consequently, TCN was re-estimated by qPCR using genomic DNA extracted from the sorted populations.

**Figure 3.** The HDAC6 3'-UTR variant leads to increased expression of eGFP in MG63 cells. (A) Schematic representation of the three eGFP-expressing transgenes used. The eGFP-GLO transgene was used as reference. LTR, long terminal repeat; EF1α, EF1α promoter; eGFP, enhanced green fluorescent protein coding region; 3’GLO, rabbit β-globin 3’-UTR; 3’HDAC6WT, wild-type HDAC6 3’-UTR; 3’HDAC6Var, variant HDAC6 3’-UTR. (B) Reverse transcribed quantitative PCR. Curves display the amplification of control RNA RNU48 in HeLa and MG63 cells (superimposed). Amplification shows that hsa-miR-433 is expressed at low level in MG63 cells and is absent in HeLa cells. The table indicates the Ct values obtained. (C) MG63 and HeLa cells were transduced with lentiviral particles containing the eGFP-HDAC6WT and eGFP-HDAC6Var transgenes. Histograms show the level of eGFP protein (P) per TCN. P/TCN ratio measures the global post-transcriptional regulation mediated by HDAC6 wild-type or variant 3’-UTRs by comparison with the referent eGFP-GLO transgene.
As shown in Figure 4A, expression of eGFP-Var or eGFP expression was analysed by flow cytometry 72 h later. hsa-miR-433, anti-hsa-miR-433 or mock transfected, and Transgene-expressing MG63 cells were transfected with hsa-miR-433 plus anti-hsa-miR-433 or mock transfected as indicated on graph legends. After 3 days, eGFP protein (P) was determined by flow cytometry on live cells. The eGFP mRNA amount (M) was determined by qPCR on reverse-transcribed total RNA extracted from each transfected cell population. RPLP0 mRNA, as well as GAPDH mRNA (data not shown), was used as internal control. The global post-transcriptional regulation (A), the relative mRNA stability (B) and the relative translation efficiency (C) were expressed, respectively, as P/TCN, M/TCN and P/M ratios. For each transgene, results were normalized to the mock transfected cells. Mann–Whitney test: n = 3; *P < 0.05.

Transgene-expressing MG63 cells were transfected with hsa-miR-433, anti-hsa-miR-433 or mock transfected, and eGFP expression was analysed by flow cytometry 72 h later. As shown in Figure 4A, expression of eGFP-Var or eGFP-GLO remained unchanged in the three conditions tested. However, a significant decrease in expression of eGFP-WT was observed in MG63 cells transfected with hsa-miR-433 compared with the mock transfected cells (39% decrease, Fig. 4A). This repressing effect of hsa-miR-433 on eGFP-WT expression was neutralized by the co-transfection of anti-hsa-miR-433 (Fig. 4A). These results therefore demonstrated that hsa-miR-433 specifically down-regulated eGFP-WT expression by targeting HDAC6 3′-UTR and that the c.281A>T variant prevented the recognition of HDAC6 3′-UTR by hsa-miR-433 in cellulo.

In order to distinguish between the two possible modes of action of hsa-miR-433, i.e. accelerated degradation of the HDAC6 mRNA or repression of its translation into a protein, functional analyses were further carried out. The quantity of eGFP mRNA (referred as M) was measured in MG63 cells expressing either eGFP-WT or eGFP-Var by quantitative RT–PCR using total RNA extracted from cells transfected by hsa-miR-433 or mock transfected. Then, the M/TCN ratio corresponding to the quantity of eGFP mRNA per transgene (Fig. 4B) and the P/M ratio corresponding to the quantity of eGFP protein produced per eGFP mRNA (Fig. 4C) were measured in each condition. When comparing eGFP expression of one transgene with that of the referent eGFP-GLO, the M/TCN and P/M ratios were indicative of relative mRNA stability and translation efficiency, respectively (12). As shown in Figure 4B and C, the post-transcriptional regulation mediated by hsa-miR-433 on eGFP-WT expression in MG63 cells was due to an increased degradation of the mRNA. Indeed whereas the translation efficiency was not significantly affected by hsa-miR-433 (Fig. 4C), the stability of the corresponding mRNA was half that measured in mock transfected cells (Fig. 4B). These results therefore demonstrated that hsa-miR-433 down-regulated eGFP-WT expression by destabilizing the mRNA bearing the wild-type HDAC6 3′-UTR. Conversely, no significant difference in mRNA stability or translation efficiency was observed in the eGFP-Var-expressing MG63 cells transfected or not by hsa-miR-433 (Fig. 4B and C). Therefore, these results showed that the c.281A>T HDAC6 variant abolishes the destabilizing effect mediated by hsa-miR-433 on eGFP-WT mRNA.

Altogether these results definitively demonstrated that hsa-miR-433 post-transcriptionally controls HDAC6 expression by accelerating the degradation of its messenger RNA and that the c.281A>T variant abolishes this regulation.

**HDAC6 protein and its deacetylating activity are increased in thymus tissue from an affected fetus**

As we demonstrated that hsa-miR-433 down-regulated HDAC6 expression in MG63 cells and that c.281A>T mutation abrogated this regulation, we next investigated whether HDAC6 expression is deregulated in tissues from a male patient bearing the c.281A>T mutation. Total proteins were extracted from thymus (the only tissue available) of the male fetus III14 (Fig. 1A) and of four control age-matched male fetuses, the pregnancy of whom was interrupted for diseases other than bone disorders. From Protein Atlas database (http://www.proteinatlas.org/) (14), HDAC6 was reported to be expressed, although weakly, in thymus. Western blot analyses with an anti-HDAC6 polyclonal antibody showed that the level of HDAC6 protein was much higher in the chondrodysplasia-affected fetus than in the four control fetuses (5.3-fold increase when compared with the mean of
the four control fetuses; Fig. 5A, upper panel, and B). These results strongly suggested that HDAC6 overexpression in the affected fetus was related to the c.*281A>T mutation. To ascertain that this difference was not due to a change in hsa-miR-433 expression, the level of hsa-miR-433 expression was determined by quantitative RT–PCR. Results in Figure 5C showed that the quantity of hsa-miR-433 in the thymus of the affected fetus was not very different, although it was lower in 3 cases, from that in the control fetuses. Therefore, we think that this weak difference of hsa-miR-433 expression could not likely account for HDAC6 overexpression, although this was not experimentally demonstrated. In conclusion, these results showed that HDAC6 expression was increased in the thymus of an affected fetus harbouring the dominant X-linked chondrodysplasia.

One substrate of HDAC6 is the acetylated α-tubulin (15). In order to evaluate the functional consequences of HDAC6 overexpression in vivo, the levels of total and acetylated α-tubulin were compared in the thymus from the affected fetus III14 and the four control fetuses. As shown in Figure 5A (second panel from the top) and D, the level of total α-tubulin was highly increased in the thymus of fetus III14 when compared with the control fetuses, a phenomenon that was already described in cells that overexpress HDAC6 (15). Concerning the level of acetylated α-tubulin, results in Figure 5A (third panel from the top) showed that it decreased by a factor 11 in the affected fetus when compared with the controls. Taken together, the acetylated/total α-tubulin ratio was diminished by a factor 7.5 in fetus III14 (Fig. 5E). Altogether our data strongly support the existence of a molecular link between the detection of the c.*281A>T variant and the increased expression of HDAC6 in tissue of a fetus affected with the new type of chondrodysplasia we have described.

Figure 5. Analysis of HDAC6, total and acetylated α-tubulin expressions in thymus from an affected fetus. (A) Total proteins extracted from thymus of fetus III14 were analysed by western blotting using antibodies raised against HDAC6, α-tubulin, acetylated α-tubulin and GAPDH. 1–4: control male fetuses; III14: affected male fetus. The proteins are as indicated on the right and their corresponding molecular weights in kDa are shown on the left. (B–E) Histograms show the expression of HDAC6 protein normalized to GAPDH (B), hsa-miR-433 expression normalized to RNU48 (C), total α-tubulin expression normalized to GAPDH (D), acetylated/total α-tubulin expression ratio normalized to GAPDH level (E) and in the thymus of different fetuses. The mean of the values obtained from the four control fetuses is as indicated in (B–E).
Functional analyses in skin fibroblasts from an affected female patient

We have shown by RT–PCR on mRNA extracted from lymphocytes of all affected females from the family under study that only the normal allele of HDAC6 was transcribed. This was in accordance with the complete bias of X chromosome inactivation observed in these cells (data not shown). Fibroblasts were obtained from skin biopsies from the right and left arms of a young affected female from the family (III16, Fig. 1) who presented with left limbs strikingly shorter than the right limbs (Fig. 6A). The left–right body asymmetry along with the presence of hyperpigmented strikes following Blaschko lines suggests the presence of a somatic mosaicism in this patient. X chromosome inactivation studies performed by testing a methylation-sensitive locus at the HUMARA gene in Xq13 (16) showed that the right arm (‘normotrophic’) fibroblasts displayed a skewed X chromosome inactivation pattern (90–10%), whereas the left arm (‘hypotrophic’) fibroblasts did not display an X chromosome inactivation bias (69–31%) (Fig. 6B). RT–PCR experiments showed that only the normal allele was expressed in the right arm fibroblasts, whereas both the normal and the mutated alleles of HDAC6 were expressed in the left arm fibroblasts (Fig. 6C). Analysis of the segregation of the HUMARA alleles showed that the 190 bp allele inherited through all affected carrier females was located on the X chromosome that was inactivated in lymphocytes and thus presumably carried the HDAC6 mutation (Fig. 6D). The same HUMARA allele was found to be on the inactive X chromosome in 90% of the right arm-derived fibroblasts (Fig. 6B1). On the other hand, in left arm-derived fibroblasts, this allele was on the inactive X chromosome in 69% of cells, and on the active X chromosome in 31% of cells (Fig. 6B2).

The levels of HDAC6 mRNA and hsa-miR-433 were determined by real-time qPCR as described above on left and right arm fibroblasts and on fibroblasts from an unaffected adult female control. The left arm fibroblasts expressed HDAC6 mRNA and hsa-miR-433 at a higher level than the right arm fibroblasts (factor 2.15 and 1.4, respectively; not statistically significant) (Fig. 7A and B). The HDAC6 protein was also found to be overexpressed in the left arm fibroblasts when compared with the right arm fibroblasts (factor 1.57; not statistically significant) (Fig. 7C). Accordingly, the acetylated α-tubulin/total α-tubulin ratio was found to be decreased in the left arm fibroblasts when compared with the right arm fibroblasts (factor 0.85; not statistically significant) (Fig. 7D). These modest increases in HDAC6 mRNA and protein and decrease of the acetylated α-tubulin/total α-tubulin ratio in the left arm-derived fibroblasts compared with the right arm-derived fibroblasts can be explained by the fact that only 31% of left arm-derived fibroblasts express the mutated allele. The right arm fibroblasts expressed the HDAC6 mRNA and the HDAC6 protein at levels similar to fibroblasts from a control female.

Altogether these data suggest that the right arm fibroblasts are representative of a ‘normal’ phenotype. On the contrary, the left arm fibroblast population that expresses both the normal and mutated HDAC6 alleles and displays an increased expression level of HDAC6 mRNA and protein reflects the disease phenotype. Thus the overexpression of HDAC6 that was observed both in the thymus of affected male III14 and in the left arm-derived fibroblasts of female patient III16 in this family is likely to be linked to the disease. The increased level of HDAC6 protein results in increased deacetylase activity. The decreased level of acetylated α-tubulin thus constitutes a biochemical phenotype that results from the presence of the HDAC6 3′-UTR variant. It can be speculated that other functions of HDAC6, particularly through deacetylation of its substrates may be augmented as well, thus causing the clinical phenotype observed in this family.

DISCUSSION

We have identified a variant (c.*281A>T) in the 3′-UTR of the HDAC6 gene that is present in all male and female affected members of a family with X-linked dominant chondrodysplasia. We have shown that this variant is located in the HDAC6 sequence matching the seed of hsa-miR-433 and totally abrogates the post-transcriptional regulation normally exerted by this microRNA on an HDAC6 3′-UTR-bearing transgene in transduction experiments. Our data indicate that hsa-miR-433 is responsible for the down regulation of HDAC6 through its interaction with the 3′-UTR of the gene since challenge experiments with a synthetic anti-hsa-miR-433 abolishes this down-regulation. We also showed that the HDAC6 protein was overexpressed in thymus of an affected male fetus (III14) and in fibroblasts expressing the mutated allele obtained from a skin biopsy of an affected female patient (III16). The striking left–right body asymmetry, characterized by hypotrophy of the left limbs and normal growth of the right limbs, along with the presence of hyperpigmented strikes following Blaschko lines, is suggestive of a somatic mosaicism of the disease expression in this patient. This phenotype was also observed to a lower degree in some other female patients of the family. We found a selective inactivation of the HDAC6 variant-bearing X chromosome in the right arm-derived fibroblasts (90–10%) and an absence of selective inactivation of this X chromosome in the left arm-derived fibroblasts (69–31%), and showed that expression of the mutated allele in the left arm-derived fibroblasts led to increased HDAC6 expression. Analysis of joint-derived cells such as chondrocytes would have been more relevant and demonstrative, but such material was not available.

All these data strongly suggest that the c.*281A>T variant in the 3′-UTR of the HDAC6 gene is the mutation that causes the disease in the family under study, and that the mechanism involved in the pathogenesis is the deregulation of HDAC6 expression due to a lack of recognition of the mutated HDAC6 3′-UTR by hsa-miR-433, therefore leading to the overexpression of the HDAC6 protein.

To date, there are only few examples showing a direct link between altered gene expression regulation and human diseases. A variant in the 3′-UTR of the SLITRK1 gene was found to create a binding site of increased affinity for hsa-miR-189 with respect to the wild-type allele that led to repression of SLITRK1 expression in patients with Tourette syndrome (17). A variant in the hsa-miR-433-matching site of the FGF20 gene constitutes a risk factor for Parkinson disease (18). Very recently mutations in the seed region of
hsa-miR-96 were identified in patients with autosomal dominant progressive hearing loss (19). Altered microRNA-based regulation of gene expression has also been found in cardiac diseases (20,21) and cancers (reviewed in 22).

HDAC6 is a deacetylase that has numerous substrates, one of which is acetylated α-tubulin. We have shown that the over-expression of HDAC6 in thymus from an affected fetus led to a strong decrease in the level of acetylated α-tubulin that is paralleled by an increased level of total α-tubulin. It is known that the level of α-tubulin acetylation acts on the dynamic of microtubules, and therefore on cell motility (23) and migration (24). The latter study showed that cells migrate over 2-
5-fold longer distances when the HDAC6 protein is overexpressed. The deacetylation of α-tubulin by HDAC6 leads to the de-polymerization of microtubules and to their subsequent destabilization (24). It is notable that affected fetuses in this family present a mis-orientation of cranio-caudal fibroblasts (2), which might result from abnormal cell migration during development, possibly caused by microtubule destabilization.

It will be interesting to test in the future the effect of the mutation, and hence of HDAC6 overexpression, on other substrates of HDAC6, such as cortactin, Hsp90, β-catenin and actin. A decreased level of acetylated cortactin was shown to increase cell motility (25). Hsp90, a member of the heat shock protein family, acts as a chaperone for diverse regulatory proteins. Docking of these proteins in the cytoplasm by Hsp90 modifies their regulation and triggers their degradation by the proteasome pathway. HDAC6 modulates the relative levels of acetylated and non-acetylated Hsp90, and therefore regulates Hsp90 function (26). The nuclear localization of β-catenin is regulated by HDAC6-dependant deacetylation (27). Actin is involved in cell morphology and migration (28).

HDAC6 interacts with the nuclear protein RUNX2 (runt-related transcription factor 2), a transcription factor that is essential for osteoblast development, thereby regulating tissue-specific gene expression (29). Loss of function mutations of RUNX2 lead to cleidocranial dysplasia in human (30) and mouse (31). HDAC4, another class II Histone deacetylase, is expressed in prehypertrophic chondrocytes, where it regulates the development of osteoblasts through its interaction with RUNX2. This interaction suppresses RUNX2 activity, which is expected to prevent chondrocyte hypertrophy and differentiation. The overexpression of HDAC4 in proliferating chondrocytes in transgenic mice leads to the inhibition of developing bones ossification and results in a Runx2 loss of function phenotype (32). All bones ossifying through an endochondral process are affected, whereas membranous ossification is not altered. Ribcage, vertebrae and the base of the skull were particularly affected, as is the case in the affected male fetuses in the family we are studying. Even more strikingly, some of the transgenic animals displayed severe foreshortening of limbs, a feature that is central in the phenotype of both affected males and females in the family we are studying.

These data show that HDAC4 is a key regulator of chondrocyte hypertrophy and of skeletal genesis. It has been shown that HDAC6 levels increase during osteoblast differentiation (29). Knowing that both HDAC4 and HDAC6 are class II HDACs, it is tempting to speculate that the overexpression of HDAC6 has a similar effect to that associated with HDAC4 overexpression. It should be noted that HDAC6−/− mice do not display any particular phenotype (33). Transgenic mice overexpressing HDAC6 have not been described yet, and it will be of special interest to produce such mice in order to study the effect of HDAC6 overexpression on skeletal development.

A further link between HDAC6 and bone development resides in the fact that the Indian hedgehog (IHH) gene is a direct target of transcription factor RUNX2, and that RUNX2 expression in proliferating chondrocytes induces IHH expression in prehypertrophic chondrocytes (34). Ihh−/− deficient mice display severe skeletal abnormalities.
characterized by a marked decrease in chondroblast proliferation and osteoblast differentiation (35). The overexpression of HDAC6 may, therefore, through its interaction with RUNX2, alter the IHH developmental pathway and thus lead to abnormal bone morphogenesis.

On the basis of data obtained in Hdac4−/−, Hdac5−/− and Hdac9−/− mice, it is proposed that class II HDACs act in diverse cell types to control hypertrophic growth in response to developmental signals (32). Hypertrophic growth of different cell types depends upon different stimuli and is controlled by distinct sets of transcription factors, such as RUNX2 in chondrocytes.

Our data, supported by the known functions of HDAC6 and other HDACs, in particular HDAC4, another class II HDAC, strongly suggest that we have probably identified the molecular cause of the new form of X-linked chondrodysplasia that we previously described (2). This would represent to our knowledge the first example of a developmental disease caused by the abrogation of the post-transcriptional regulation normally exerted by a microRNA on its target gene. The new form of the chondrodysplasia we have described has not been found in another family so far. Therefore, we have not been able to replicate the finding of a HDAC6 mutation in a second family with a phenotype resembling that found in the patients from the family we have been studying. Our data should now be supported by the creation of a mouse model that reproduces the mutation identified here or that overexpresses HDAC6 by classical transgenesis.

MATERIALS AND METHODS

Lentiviral plasmid constructs

The pTRIPdeltaU3-EF1α-GFP (pTRIP-eGFP) lentiviral plasmid was a gift of Pierre Charneau (Institut Pasteur, Paris, France). The eGFP expression is driven by the constitutive EF1α promoter. The pTRIP-0 and pTRIP-eGFP-GLO plasmids were constructed as described previously (12). The pTRIP-eGFP-HDAC6WT and pTRIP-eGFP-HDAC6VAR plasmids were constructed as follows. The human HDAC6 3′-UTR fragment was gel purified and inserted into the pGEM-T plasmid (Promega) according to the manufacturer’s instructions. The integrity of the WT or c.281A>T HDAC6 3′-UTR was then assessed by DNA sequencing. The resulting plasmids (pGEM-HDAC6WT and pGAM-HDAC6VAR) were digested with XhoI and KpnI, each HDAC6 3′-UTR fragment was gel purified and inserted into the XhoI- and KpnI-digested pTRIP-eGFP plasmid.

Cell lines and miRNA transfection

The adenocarcinoma HeLa and osteosarcoma MG63 cell lines were grown in medium with 10% fetal calf serum and antibiotics. The hsa-miR-433 and anti-hsa-miR-433 were obtained from Thermo Scientific Dharmacon. Small RNAs were transferred in the target cells using INTERFERin (Ozyme).

Lentiviral production, titration and cell transduction

Lentiviral particles were produced as described previously (12). Infectious lentiviral particles were added to the target cells in their respective culture medium and incubated for 24 h. Cells were then washed and grown in the presence of medium for 6 days before experimental use.

Analysis of cells by flow cytometry

One week after transduction, cells were washed in PBS, detached with trypsin/EDTA, collected and analyzed by FCM using an EPICS XL flow cytometer (Coulter) and the Expo 32 analysis software as described previously (12).

PCR sequencing

All genes were amplified by PCR reaction using Taq gold (Applera) and sequenced using Big dye (Applera). The amplification was done in parallel on patients II7, III14 and a control. Primers used for PCR and sequencing, as well as experimental conditions can be obtained from the authors upon request.

Real-time qPCR and RT–PCR

Total DNA was extracted from cells using the Nucleospin Tissue (Macherey-Nagel). Total RNA was extracted from cells or tissues using Trizol. Real-time qPCR amplifications were performed as described previously (12). Succinctly, PCR reactions contained 1X iQ™ SYBR® Green Supermix (Biorad), the two primers (see Supplementary Material for details) and either 50 ng of total DNA or cDNAs from 100 ng of reverse-transcribed total RNA. The Albumin, RPLP0 or α-tubulin mRNA served as internal control for normalization. The GAPDH mRNA was used as a second internal control.

Real-time qPCR of RNU-48 (Applera) and hsa-miR-433 was performed with TaqMan assays using specific kits from Applera.

Subsequent data analyses were performed using the M×4000 Multiplex Quantitative PCR System equipped with Version 4.2 software (Stratagene).

Array-CGH analysis

Genomic DNA was extracted from peripheral blood lymphocytes or tissues of patients II7 and III14 and of a reference individual, and labelled differentially with Cytomix 3 and 5 (Bioprint_DNA labelling system from Invitrogen, Cytomix 3 and 5 from Amersham). Microarray experiments were performed on DNA microarrays 44A (patient II7) or 244A (patient III14) (Agilent Technologies). Colour reverse experiments (Cytomix 3 and 5 swapping) were performed. Microarrays were scanned with an Agilent scanner G2565BA. Hybridization data analysis was carried out with softwares from Agilent Technologies: Feature Extraction (v.9.5.3.1) for...
the ratio calculation and CGH Analytics (v.3.4.27) for the localization of chromosomal imbalances.

**Total proteins extraction and western blot analyses**

Total proteins were extracted from tissues or cells by using RIPA plus protease inhibitors (Sigma Aldrich). Protein quantity was measured with a Bio-Rad assay. Equivalent amounts of proteins were separated by SDS–PAGE, transferred to BA85 nitrocellulose membrane (Schleicher and Schuell), and analyzed by two colour western blotting with antibodies raised against HDAC6 (Santa Cruz H-300), α-tubulin (Sigma Aldrich T-6074), acetylated α-tubulin (Sigma Aldrich T-6794), GAPDH (Santa Cruz SC-45718). Blots were blocked with Odyssey Blocker and incubated with primary antibodies, followed by IR-labelled secondary antibodies (anti-mouse for acetylated α-tubulin and anti-rabbit for α-tubulin, eGFP, GAPDH, HDAC6). Signals were detected and quantified using the Odyssey infrared imaging system.

**Statistical analysis**

Data are mean ± standard deviation (SD) from three independent experiments. The non-parametric Mann–Whitney test was used for the comparison of two values with small sample size. *P* < 0.05 was considered statistically significant. All analyses were performed using Stata 7.05 E Software.

**X chromosome inactivation analysis**

X chromosome inactivation pattern was established using a test based on methylation analysis of HpaII and CfoI restriction sites located close to the androgen receptor HUMARA (CAG)n repeat (15). Briefly, DNA was extracted from fibroblasts cultivated from left and right arm skin biopsies from patient III16. 300 ng of DNA, either digested with restriction enzymes HpaII and CfoI or not digested, was submitted to PCR with primers designed to amplify a fragment containing a polymorphic (CAG)n repeat of the HUMARA gene. The PCR products were thereafter fractionated by capillary electrophoresis on an ABI 3130 DNA analyser (Applera). Fragment sizes were determined using the GeneMapper software (Applera).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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**REFERENCES**


